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Deletion mutagenesis using an 'M13 splint': the N-terminal structural domain of tyrosyl-tRNA synthetase (*B. stearothermophilus*) catalyses the formation of tyrosyl adenylate

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The X-ray crystallographic structure of tyrosyl-tRNA synthetase (TyrTS) comprises only the N-terminal 320 amino acids of the molecule as the C-terminal 99 amino acids are poorly ordered in the crystal. A new technique, employing a single-stranded M13 splint, has been used to direct a deletion in the cloned gene of TyrTS so as to remove the disordered C-terminal region. We find that the truncated enzyme catalyses the formation of tyrosyl adenylate with unchanged k_{cat} and K_m values and the crystallographic model must therefore include all the binding and catalytic residues involved in tyrosine activation. However, the truncated enzyme no longer binds tRNA^{Tyr} or transfers tyrosine to tRNA^{Tyr}. This indicates that the structural division of TyrTS is equally a functional one: the N-terminal structural domain catalyses tyrosine activation while the disordered C-terminal domain carries major determinants in tRNA binding.
Key words: deletion mutagenesis/double domain structure/ M13/tyrosyl-tRNA synthetase

Introduction

Tyrosyl-tRNA synthetase (TyrTS) is a dimeric molecule of 419 amino acids (Winter *et al.*, 1983) which catalyses the activation of tyrosine with ATP and the subsequent transfer of tyrosine to tRNA^{Tyr} (Loftfield, 1972; Fersht and Jakes, 1975). The X-ray crystallographic structure of the enzyme encompasses only the N-terminal two-thirds of the sequence as the C-terminal third is disordered in the crystal (Bhat *et al.*, 1982). Binding sites for tyrosine, ATP and tyrosyl adenylate (Monteilhet and Blow, 1978) have been located in the crystallographic model as well as possible tRNA contacts fringing the active site (Bosshard *et al.*, 1978). Furthermore, the precise role of a cysteine residue in catalysis and ATP binding has been directly assessed by utilising site-directed mutagenesis of the enzyme (Winter *et al.*, 1982; Wilkinson *et al.*, 1983). However, the contribution, if any, of the disordered C-terminal region of the enzyme to substrate binding or catalysis is not clear. Attempts to remove this region by proteolysis result in cleavage within the N-terminal domain, probably at Arg-157, and loss of enzyme activity (Winter *et al.*, 1979; G. Winter, unpublished results). Since TyrTS has been cloned into the plasmid pBR322 (Barker, 1982) and thence into the bacteriophage M13 (Winter *et al.*, 1982) and is expressed well in *Escherichia coli*, we can remove the carboxy terminus at the gene level. We have achieved this by a new technique in which we deleted a restriction fragment from a partial duplex of the single-stranded TyrTS gene and a single-stranded M13 splint clone (Figure 1). The truncated (319 amino acids) enzyme was expressed and enzyme assays show that it catalyses tyrosine activation but not aminoacylation of tRNA^{Tyr}.

Results

Construction of truncated TyrTS

The agarose gel in Figure 2 illustrates the annealed hybrid and its digestion with *Hinf*I: the digested product runs more slowly than the hybrid. More extensive *Hinf*I digestion occasionally resulted in substantial degradation of the parental and annealed single strands, possibly due to minor cleavages of *Hinf*I on local double-stranded structures. Ligation and transformation of 0.2 μg of the digested duplex yielded 150 plaques, and dideoxy T-tracks (Sanger *et al.*, 1977) using a synthetic oligodeoxynucleotide primer (Figure 1) revealed that, of 24 plaques, 13 corresponded to the truncated gene and 11 to the entire TyrTS gene. The complete sequence of one clone of the truncated gene proved that there had been no other changes and that the truncated enzyme was therefore Met 1-Arg 317 with a C-terminal Tyr-Ala.

Activity and subunit structure of truncated enzyme

tRNA charging activity could not be detected in the truncated enzyme and is at least 3000 times lower than in the native enzyme which was assayed in parallel as described in Wilkinson *et al.* (1983). The k_{cat} (turnover number) and K_m values for the tyrosine and ATP substrates in the pyrophosphate exchange were essentially the same as in the native enzyme (Table I) assayed in parallel. The loss of tRNA charging is probably due to lack of tRNA binding; no binding of [^{14}C]Tyr-tRNA^{Tyr} (1.1 μM) to the truncated TyrTS (0.7 μM) could be detected using a filter binding assay as in Buonocore and Schlessinger (1972) whereas, in a parallel experiment, intact TyrTS bound well to [^{14}C]Tyr-tRNA^{Tyr}. The truncated

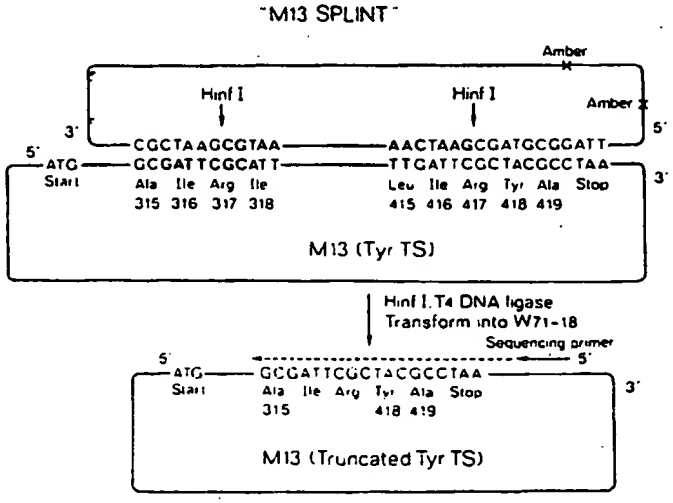

Fig. 1. Scheme to illustrate the deletion of a *Hinf*I fragment from the TyrTS gene cloned in the single-stranded bacteriophage M13.

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Fig. 2. *Hinf*I digestion of annealed clones fractionated on an 0.7% agarose gel and stained with ethidium bromide. (A) 'M13 splint'; (B) M13 (TyrTS); (C) annealed hybrid; (D) *Hinf*I-digested hybrid. The arrow marks the digested hybrid.

Table I. Effect of deleting C terminus of TyrTS on k_{cat} and K_m for ATP and tyrosine in the pyrophosphate exchange

		Intact TyrTS	Truncated TyrTS
k_{cat}	(ATP)	8 s^{-1}	6.9 s^{-1}
K_m	(ATP)	1.35 mM	1.38 mM
k_{cat}/K_m	(ATP)	5900 s^{-1} mol^{-1}	5000 s^{-1} mol^{-1}
k_{cat}	(tyrosine)	5.5 s^{-1}	4.6 s^{-1}
K_m	(tyrosine)	1.8 μM	2.4 μM

K_m (ATP) was measured at 50 μM tyrosine and K_m (tyrosine) at 2 mM ATP.

enzyme was found to have a subunit mol. wt. of ~36 000 on SDS-polyacrylamide gels (Laemmli, 1970) (not shown). This compares well with the mol. wt. of 36 324 calculated from the amino acid sequence. By gel filtration (Figure 3) the truncated enzyme was found to have a mol. wt. of 70 000 ± 2000 and is therefore a dimer.

Discussion

Use of M13 splint to construct deletions

In principle, the construction of proteins truncated at their carboxy termini could be achieved by deleting the corresponding portion of the gene or by introducing a premature termination codon via primer-directed mutagenesis (Hutchinson *et al.*, 1978). The deletion approach should be superior in that termination codons can revert and may be read through to a limited extent, even in tightly non-suppressing hosts (Kelly and Joyce, 1983). In this particular case, the convenience of two *Hinf*I sites flanking the disordered region at the C terminus permitted a deletion strategy. We have developed a method in which a single *Hinf*I fragment was deleted from the TyrTS gene with an M13 splint, despite the presence of >50 *Hinf*I sites in the recombinant M13 clone.

The hybridisation between the TyrTS gene (wild-type phage) and the splint (amber phage) was driven by an excess of splint clone (3/1). By transfecting into a non-suppressor strain (W71-18) the splint clones were removed microbiologically. The transformation/ligation efficiency of the cut

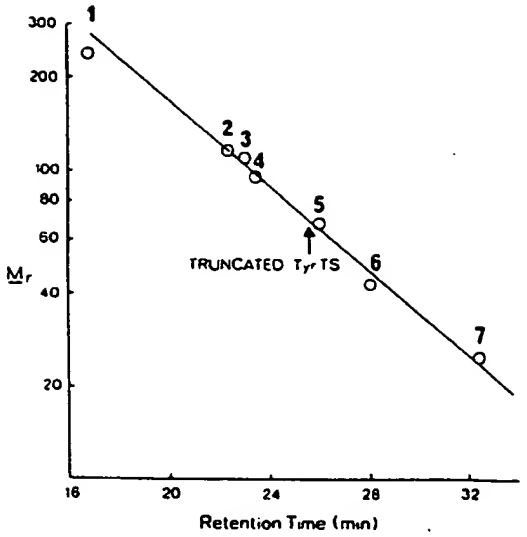

Fig. 3. Determination of native mol. wt. and hence subunit composition of truncated TyrTS. The proteins were sized by gel filtration using an LKB TSK-G 3000SW column (7.5 x 600 mm), flow rate 0.7 ml/min, in 0.1 M potassium phosphate (pH 7.0) and 0.2 M sodium chloride. Proteins used for calibration were: 1, pyruvate kinase (tetramer) (mol. wt. 237 000) 2, pyruvate kinase (dimer) (118 000); 3, valyl-tRNA synthetase (110 000); 4, TyrTS (95 000); 5, bovine serum albumin (68 000); 6, ovalbumin (43 000); 7, chymotrypsinogen A (25 000).

duplex was low and our yields of plaques were greatly improved (>70 times) by transfecting W71-18 according to Hanahan (1983).

The use of M13 splints to make deletions is obviously constrained by convenient restriction sites and is not suitable for enzymes which are inhibited by single-stranded DNA (e.g., *Alu*I) or which cut single-stranded DNA (e.g., *Hae*III). Otherwise this technique should be general, although the final yields of the deletion clone could well depend on the stability of the cut duplex during ligation. Hence, in the cut duplex, the degree of overlap with splint on either side of the restriction site could be important: in our example there are 66 bp to one side and 150 bp to the other.

Activity of truncated enzyme

The result that the N-terminal domain catalyses tyrosine activation is not surprising as the binding sites for tyrosine, ATP and tyrosyl adenylate are located in this domain of the X-ray crystallographic structure (Monteilhet and Blow, 1978; Bhat *et al.*, 1982). The k_{cat} and K_m values, very similar in both the complete and truncated enzymes, indicate that the disordered C-terminal domain makes no contribution to tyrosine activation. The effect of excising the C-terminal domain is to abolish $tRNA^{Tyr}$ binding and hence the transfer of tyrosine to tRNA: the fact that the truncated enzyme is dimeric confirms the location of the inter-subunit contacts in the N-terminal region (Bhat *et al.*, 1982) and shows that the loss of tRNA binding is not due to dissociation of the subunits. The crystallographic disorder in the tRNA binding domain of TyrTS may have a parallel with tobacco mosaic virus. In the crystallographic structure of the intact virus (Stubbs *et al.*, 1977), the coat protein subunit binds to RNA via an α-helix. However, in the crystallographic structure of the protein disc (Bloomer *et al.*, 1978), in which the RNA is absent, the helix becomes flexible because of thermal motion (Jardetzky *et al.*, 1978) and the structure is disordered in this

region.

Overall, our results indicate that the TyrTS has a double-domain structure in which the N-terminal domain catalyses the activation of the amino acid and the C-terminal domain binds tRNA. Such an arrangement is surely true for the homologous methionyl-tRNA synthetase (Risler *et al.*, 1981; Barker and Winter, 1982; Zelwer *et al.*, 1982) and possibly for the alanyl-tRNA synthetase in which an N-terminal proteolytic fragment of AlaTS catalyses the activation of alanine but not its transfer to tRNA (Putney *et al.*, 1981a, 1981b). The independence of the amino acid activation and aminoacylation reaction, also evident in temperature-sensitive mutants of valyl-tRNA synthetase (Eidlic and Neidhardt, 1965) and tryptophanyl-tRNA synthetase (Nazario *et al.*, 1971), suggests that the double-domain structure could be widespread among aminoacyl-tRNA synthetases.

Materials and methods

Construction of truncated TyrTS

An M13 splint clone, with insert in an orientation opposite to that of the TyrTS gene in M13mp93 (Winter *et al.*, 1982) was prepared by cloning a suitable 516-bp *Hae*III fragment (see Figure 1) into the vector M13mp7 (Messing *et al.*, 1981). 2 ml cultures of *E. coli* strain JM101 (Messing, 1979) were used to prepare single-stranded M13 template as in Winter and Fields (1980): 1 μg of the single-stranded TyrTS gene and 3 μg of single-stranded splint were hybridised in 14 μl 50 mM NaCl, 7 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$ at 68°C for 1 h and cooled slowly to room temperature. The partial duplex was digested with 0.25 U *Hinf*I (New England Biolabs) for 1 h, the digest checked for completeness on an 0.7% submarine agarose gel and terminated by heating at 65°C. 0.2 μg of the digested hybrid was ligated at 15°C for 5 h in 10 μl of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol and 0.4 U T4 ligase (a gift from S. Fields), and used to transform the non-suppressor *E. coli* strain W71-18 (Winter *et al.*, 1981) as in Hanahan (1983). Plaques were screened by dideoxy T-tracks (Sanger *et al.*, 1977) using a 17-mer synthetic oligodeoxynucleotide sequencing primer (5′ TTACTATGCCCAGCGC 3′) and a clone with the required deletion was then fully sequenced using three other synthetic oligodeoxynucleotide primers. These primers were complementary to the M13 plus strand and were staged at ~300 base intervals towards the 5′ end of the TyrTS gene (P. Carter, unpublished).

Enzyme purification, kinetic and binding assays

Both wild-type and truncated TyrTS were prepared by infecting an *E. coli* host W71-18 (Winter *et al.*, 1981) with the recombinant M13 phage as described in Wilkinson *et al.* (1983). After sonication, the soluble cell extract was purified as in Wilkinson *et al.* (1983). Alternatively the extract was heated to 55°C for 30 min, centrifuged at 10 000 *g* and the enzyme in the supernatant purified on a Pharmacia FPLC monoQ column. Active site titration, pyrophosphate exchange, tRNA charging (Fersht and Jakes, 1975; Wilkinson *et al.*, 1983) and tRNA binding (Buonocore and Schlessinger, 1972) were performed as described previously.

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